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Effects of alkaloid-rich extract from *Mitragyna speciosa* (Korth.) Havil. on naloxone-precipitated morphine withdrawal symptoms and local field potential in the nucleus accumbens of mice

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ABSTRACT

Ethnopharmacological relevance: *Mitragyna speciosa* (Korth.) Havil. (*M. speciosa*) is among the most well-known plants used in ethnic practice of Southeast Asia. It has gained increasing attention as a plant with potential to substitute morphine in addiction treatment program. However, its action on the central nervous system is controversial.

Aim of the study: This study investigated the effects of *M. speciosa* alkaloid extract on naloxone-precipitated morphine withdrawal and neural signaling in the nucleus accumbens (NAc, brain reward center) of mice.

Materials and methods: The effects of *M. speciosa* alkaloid extract and mitragynine, a pure major constituent, on naloxone-precipitated morphine withdrawal were examined. Male Swiss Albino (ICR) mice were rendered dependent on morphine before injection with naloxone, a nonspecific opioid antagonist, to induce morphine withdrawal symptoms. The intensity of naloxone-precipitated morphine withdrawal was assessed from jumping behavior and diarrhea induced during a period of morphine withdrawal. To test possible addictive effect of *M. speciosa* alkaloid extract, mice were implanted with intracranial electrode into the NAc for local field potential (LFP) recording. Following *M. speciosa* alkaloid extract (80 mg/kg) and morphine (15 mg/kg) treatment, LFP power spectra and spontaneous motor activity were analyzed in comparison to control levels.

Results: One-way ANOVA and multiple comparisons revealed that *M. speciosa* alkaloid extract (80 and 100 mg/kg) significantly decreased the number of jumping behavior induced by morphine withdrawal whereas mitragynine did not. Additionally, *M. speciosa* alkaloid extract significantly decreased dry and wet fecal excretions induced by morphine withdrawal. LFP analysis revealed that morphine significantly decreased alpha (9.7–12 Hz) and increased low gamma (30.3–44.9 Hz) and high gamma (60.5–95.7 Hz) powers in the NAc whereas *M. speciosa* alkaloid extract did not. Spontaneous motor activity was significantly increased by morphine but not *M. speciosa* alkaloid extract.

Abbreviations: AP, Anteroposterior; ANOVA, Analysis of variance; BW, Body weight; CNS, Central nervous system; DV, Dorso-Vental; FFT, Fast Fourier transform; HPLC, High performance liquid chromatography; ICLAS, International Committee on Laboratory Animal Science; LAAM, levo-alpha-acetylmethadol; LC-MS, liquid chromatographic mass spectrometry; LFP, Local field potential; ML, Medio-Lateral; NAc, Nucleus accumbens; PSD, Power spectral density; TLC, Thin layer chromatography; S.E.M., Standard error of the mean

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Conclusions: Taken together, *M. speciosa* alkaloid extract, but not mitragynine, attenuated the severity of naloxone-precipitated morphine withdrawal symptoms. Neural signaling in the NAc and spontaneous motor activity were sensitive to morphine but not *M. speciosa* alkaloid extract. Therefore, treatment with the *M. speciosa* alkaloid extract may be useful for opiate addiction treatment program.

1. Introduction

Mitragyna speciosa (Korth.) Havil. (*M. speciosa*), locally called Kratom in Thailand, is a member of the Rubiacaea plant family. It has been used as a medicinal plant for centuries (Jansen and Prast, 1988; Suwanlert, 1975). In modern studies, four alkaloids namely mitragynine, 7-hydroxymitragynine, corynantheidine and speciociliatine appeared to act on opioid receptor (Takayama et al., 2002). However, kratom consumption for opium-like effect was previously reported (Suwanlert, 1975). In Malaysia, there is a record that kratom was used as an affordable opium substitute (Burkill, 1935). However, the efficacy of kratom use as an opium substitute has not been clearly elucidated. There were still many questions needed to be answered in scientific ways.

The antinociceptive effects of this plant are well known and have been confirmed by many studies. The main component of the *M. speciosa* extract, mitragynine, has consistently exhibited antinociception centrally *via* the descending noradrenergic and serotonergic systems (Matsumoto et al., 1996) by acting on opioid receptors (Thongpradichote et al., 1998). However, the selectivity of mitragynine for opioid receptor subtypes is different from that of morphine (Matsumoto et al., 1996; Thongpradichote et al., 1998). In addition, the action of mitragynine on other physiological systems such as gastric acid secretion is also mediated *via* opioid receptors (Tsuchiya et al., 2002). These consistent data confirmed morphine-like actions of the plant and seemed to support an original idea that this plant could replace morphine in treatment programs (Jansen and Prast, 1988). Mitragynine was demonstrated to inhibit morphine withdrawal successfully *in vitro* (Watanabe et al., 1997). In particular, mitragynine was found to attenuate withdrawal syndrome in morphine-withdrawn zebrafish (Khor et al., 2011). These findings appeared to suggest that among many *M. speciosa* alkaloid components, mitragynine has gained the most attention as a potential morphine substitute. However, it remained to be examined whether mitragynine or crude *M. speciosa* alkaloid extract would show therapeutic effect for opiate withdrawal treatment.

Mitragynine is a major indole alkaloid of *M. speciosa* shown to have an antinociceptive action. However, 7-hydroxymitragynine, a minor constituent of this plant, also exhibits an even higher potency than mitragynine both *in vitro* (Takayama et al., 2002) and *in vivo* (Matsumoto et al., 2004a). Moreover, other alkaloid constituents of the plant may also exert potent therapeutic effects. Previously, *M. speciosa* alkaloid extract was found to act in the central nervous system (CNS) and produce antidepressant-like action *in vivo* models (Kumarnsit et al., 2007). Therefore, mode of CNS action of *M. speciosa* alkaloid extract has been tested extensively. Ultimately, its actions in addition to opioidergic mechanisms were also evidenced. The *M. speciosa* alkaloid extract was found to produce electroencephalographic patterns in the frontal and parietal cortices similarly to that of fluoxetine, a standard antidepressant drug (Cheaha et al., 2015b). These findings suggest other mode of *M. speciosa* action and possibility to apply this extract as an antidepressant-like compound.

From some qualitative studies in human, kratom is considered addictive with its patterns of use and symptoms (Saingam et al., 2013; Ahmad and Aziz, 2012). This was found as a major concern. However, classical drugs of addiction are known to act on the mesolimbic dopamine pathway as a reward system (Seiden et al., 1993; Koob, 1992). It remained to be examined whether the *M. speciosa* extract also has actions on the brain reward areas.

Opiate substances are known to produce dependence and the abrupt cessation causes withdrawal syndrome (Gold et al., 1978). Acute morphine withdrawal can also be induced in animal models. It emerges in morphine-dependent animals immediately after the administration of naloxone, a nonspecific opiate antagonist. Opioid receptor agonists including methadone have been used to relieve withdrawal symptoms (McMillan et al., 1976) but methadone also has side effects and can cause withdrawal by itself (Beswick et al., 2003; Gossop et al., 1989, 1987). Later, levo-alpha-acetylmethadol (LAAM), a synthetic μ opioid receptor agonist, was also found to have some potential advantages with better preference (Trueblood et al., 1978). However, use of LAAM was believed to be linked with arrhythmia (Clark et al., 2002). For more effective treatment of the opiate withdrawal syndrome, partial or non-opiate substances have also been sought for treatment of opiate addiction.

The *M. speciosa* alkaloid extract was hypothesized to reduce the intensity of opioid withdrawal. The effects of *M. speciosa* alkaloid extract on morphine withdrawal symptoms were tested. Jumping behavior and diarrhea during withdrawal period were measured as indicative data to represent the intensity of morphine withdrawal symptoms in mice. In this study, pure mitragynine and *M. speciosa* alkaloid extract were used for treatment of morphine withdrawal symptoms. In addition, addictive potential of *M. speciosa* alkaloid extract was examined. Following treatment with *M. speciosa* alkaloid extract, patterns of local field potential (LFP) in the nucleus accumbens, a brain reward center, and spontaneous motor activity were evaluated.

2. Materials and methods

2.1. Plant materials

Young leaves of *M. speciosa* Korth (Rubiaceae) were collected from natural sources in Songkla Province, Thailand. Plant materials were identified by Dr. Niwat Keawpradub, the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand where the herbarium voucher specimens (no. PCOG/MS001-002) have been deposited. The use of plant materials was approved by the Ministry of Agriculture of Thailand and was restricted to research purposes only.

2.2. Extraction and analysis of alkaloid extract from M. speciosa and mitragynine

An *M. speciosa* alkaloid-rich extract and mitragynine were prepared as described in previous studies (Cheaha et al., 2015a). The same batch of *M. speciosa* alkaloid-rich extract was also used for the present study within the same time period. For *M. speciosa* alkaloid-rich extract, young leaves were dried at 45–50 °C, powdered and macerated with methanol. The filtrate was evaporated *in vacuo*. The residue was dissolved in 10% aqueous acetic acid, filtrated and washed with petroleum ether, then made into alkaline (pH 9) with 25% ammonia solution and extracted with chloroform. The combined chloroform extracts that were washed, dried over anhydrous sodium sulphate and evaporated gave 0.25% of dry crude alkaloid extract. The extract was analyzed by using high performance liquid chromatography (HPLC). Mitragynine was used as a standard. HPLC analyses revealed the dominant peak of *M. speciosa* alkaloid-rich extract with the same retention time to mitragynine (Supp. Figs. 1 and 2, inset).

For mitragynine isolation, an aliquot (2.5 g) of alkaloid extract was subjected to silica gel column chromatography, eluted with 5% methanol in chloroform to obtain a major alkaloid (1.27 g), which appeared as a single spot on thin layer chromatography (TLC) analysis (four solvent systems). According to the TLC analysis, mitragynine was found to constitute about 60% of the crude alkaloid extract.

The pure mitragynine was identified by using liquid chromatographic mass spectrometry (LC-MS). A solution was run through LC-MS (Waters 2690 liquid chromatograph (Milford, MA, USA)) coupled with a mass spectrometer LCT™ (Micromass, Manchester, UK) at the Scientific Equipment Center, Prince of Songkla University. Confirmation of *M. speciosa* alkaloid extract was performed by using mitragynine as a standard. The spectrum of the *M. speciosa* alkaloid extract revealed masses m/z of 397.2, 398.2 and 399.2 (Supp. Fig. 1). The spectrum of pure mitragynine had a single mass m/z of 399.0 (Supp. Fig. 2). They were identified as mitragynine, a major component of *M. speciosa* compared with the obtained spectral data of previous published (Janchawee et al., 2007; Houghton et al., 1991; Shellard et al., 1978).

2.3. *Chemicals*

The following drugs were used: morphine sulphate (Zentiva, Hlohovec, Slovakia), naloxone hydrochloride (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The drugs were dissolved in sodium chloride solution (0.9% w/v) and given to animals in a volume of 5 ML/kg. The *M. speciosa* alkaloid extract was dissolved in co-solvent (Tween80: propyleneglycol: H_2O at a 1:4:4 ratio). A ball tipped stainless steel gavage needle was used for oral feeding.

2.4. *Animals*

This study was carried out in accordance with guidelines of the European Science Foundation (Use of Animals in Research 2001) and International Committee on Laboratory Animal Science, ICLAS (2004). The experimental protocols described in the present study were approved and guided by the Animals Ethical Committee of the Prince of Songkla University for care and use of experimental animals. All efforts were made to minimize animal suffering and to reduce the number of animals used. Male Swiss albino ICR mice (7–8 weeks old) used in each experiment were bred at the animal house of the Prince of Songkla University. They were housed in a group of 10 mice per cage (20 × 25 × 35 cm) and maintained under 12/12 dark/light cycle (lights on at 0600 a.m.) and controlled temperature (22 °C). Standard commercial food pellets and filtered water were available *ad libitum*. All the tests were performed between 8.00 a.m. and 4.00 p.m.

There were 2 different sets of animals for 2 main experiments. The first set was rendered dependent on morphine for testing the effects of *M. speciosa* alkaloid extract on morphine withdrawal symptoms ($n = 10–12$). Animals in the second set were anesthetized for electrode implantation and used for electrical brain wave study ($n = 6–8$).

2.5. *Development of morphine dependence*

Mice were rendered dependent on morphine using the method previously described (Marshall and Grahame-Smith, 1971). Briefly, morphine sulphate was injected (s.c.) 3 times daily at 08:00, 12:00 and 16:00 (50, 50 and 75 mg/kg, respectively) for 3 days. On day 4, only a single morning dose of morphine (50 mg/kg) was injected before naloxone injection.

2.6. *Observation of morphine withdrawal symptoms*

Withdrawal signs were precipitated by injection of naloxone (1.5 mg/kg, i.p.) 2 h after the final injection of morphine. Immediately after naloxone injection, animals were placed individually on filter paper in an observable cylindrical plastic container (15 cm in diameter and 50 cm in height). The behavior of animals was recorded by using a digital video camera. Fecal materials excreted during a 30-min period of withdrawal were measured. Jumping behavior was used as an experimental index of the central morphine withdrawal syndrome whereas fecal excretion reflected a peripheral withdrawal syndrome (Broseta et al., 2002).

2.7. *M. speciosa alkaloid extract treatments*

One hour before the induction of withdrawal with naloxone, mice were given a single oral administration of either sodium chloride solution (0.9% w/v), mitragynine (30, 90 and 120 mg/kg) or the *M. speciosa* alkaloid extract (20, 40, 60, 80 and 100 mg/kg). One effective dose of *M. speciosa* alkaloid extract (80 mg/kg) was selected for a study of LFP and spontaneous motor activity.

2.8. *Surgery for LFP electrode implantation*

Method of electrode implantation was also previously described (Cheaha et al., 2015a; Reakkamnuan et al., 2015). Briefly, animals were anesthetized by injection intramuscularly of a mixture of 150 mg/kg ketamine (Calypsol, Gedeon Richter Ltd., Budapest, Hungary) and 15 mg/kg xylazine (Xylavet, Thai Maji Phamaceutical co., Ltd., Bangkok, Thailand). Then, the animal's head was mounted in a stereotaxic frame. Local analgesic, lidocaine (Locana, L.B.S. Laboratory Ltd., Part., Bangkok, Thailand) was applied to the exposed tissue of the head. An incision was made at the midline to expose the skull. The silver wire electrodes (A-M system, Sequim, WA, USA) with bare diameter of 0.008 in. (Coated-0.01 in.) were stereotaxically positioned on the left nucleus accumbens (NAc) (AP: + 0.7 mm, ML: 1.3 mm, DV: 4.7 mm) according to mouse brain atlas (Franklin and Paxinos, 1998). The reference and ground electrodes were placed at midline overlying the cerebellum. Additional holes were drilled for stainless steel anchor screws. Dental acrylic (Unifast Trad, GC Dental Industrial Corp., Tokyo, Japan) was used to secure all electrodes on the skull. Following the surgery, animals were orally given an analgesic drug, 5 mg/kg meloxicam (Hofbic, Osoth Inter Laboratories Co., Ltd., Chonburi, Thailand). The antibiotic ampicillin (General Drug House Co., Ltd., Bangkok, Thailand) was applied intramuscularly (100 mg/kg) once a day for 3 days to prevent infection and allowed them to recover for at least 7–10 days

2.9. *LFP signals acquisition and analysis*

Methods in details of signal recoding and analysis were described in the previous study (Cheaha et al., 2015a; Reakkamnuan et al., 2015). Briefly, all signals from animal electrodes were amplified with a low-pass 1 kHz, high-pass 0.3 Hz and digitized at 2 kHz by a PowerLab 16/35 system (AD Instruments, Castle Hill. NSW, Australia) with 16-bit A/D, and stored in a PC through the LabChart 7 pro software. For offline analysis, recorded files were overviewed by using visual inspection and only noise-free signals were used for the analysis. Fifty Hz notch filtering was applied to remove the noise from power line artifacts. To avoid 50 Hz noise, the signals from 45 to 60 Hz were excluded from the further analysis. The LFP signals were processed through 1–100 Hz band-pass digital filter.

For the frequency analysis, the digitized data were assessed by analyzing the power spectral density (PSD) generated by LabChart software using the Fast Fourier Transform (FFT) algorithm. The setup conditions for FFT calculation are Hanning window cosine with 50% window overlap, and frequency resolution of 0.976 Hz. Then, the PSD in each frequency bin was expressed as percentage of total power. The power was averaged across 6 discrete frequency bands: theta, 4–8 Hz; alpha, 9.7–12 Hz; beta1, 13.6–18.0 Hz; beta2, 19.5–29.3 Hz; gamma1 or low gamma, 30.3–44.9 Hz; gamma2 or high gamma 60.5–95.7 Hz.

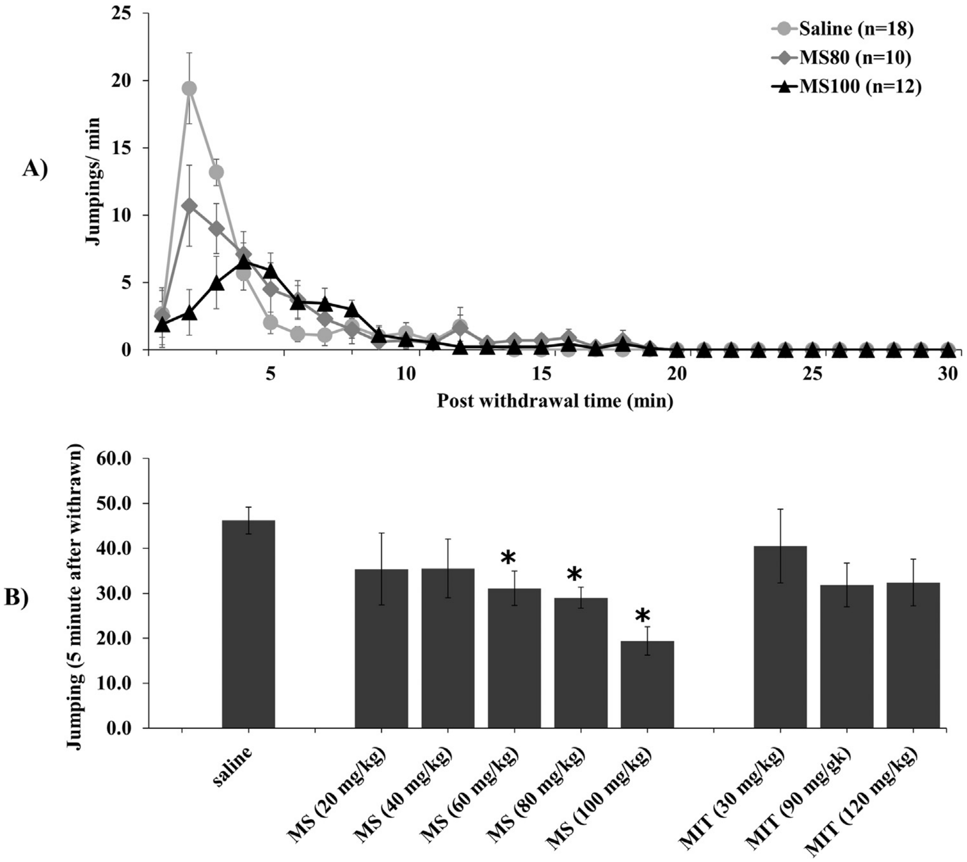

Fig. 1. Effects of *M. speciosa* alkaloid extract (MS) and mitragynine (MIT) on jumping behavior induced by naloxone-precipitated morphine withdrawal (n = 10–12). Jumping numbers of animals in control and *M. speciosa* alkaloid group (80 and 100 mg/kg) were counted minute-by-minute (A). Effects of *M. speciosa* alkaloid extracts and pure mitragynine on jumping behavior during the first 5-min period following morphine withdrawal are shown in comparison to control level (B). Representative data are expressed as means ± S.E.M. $^{*}P < 0.05$ for comparisons with control levels.

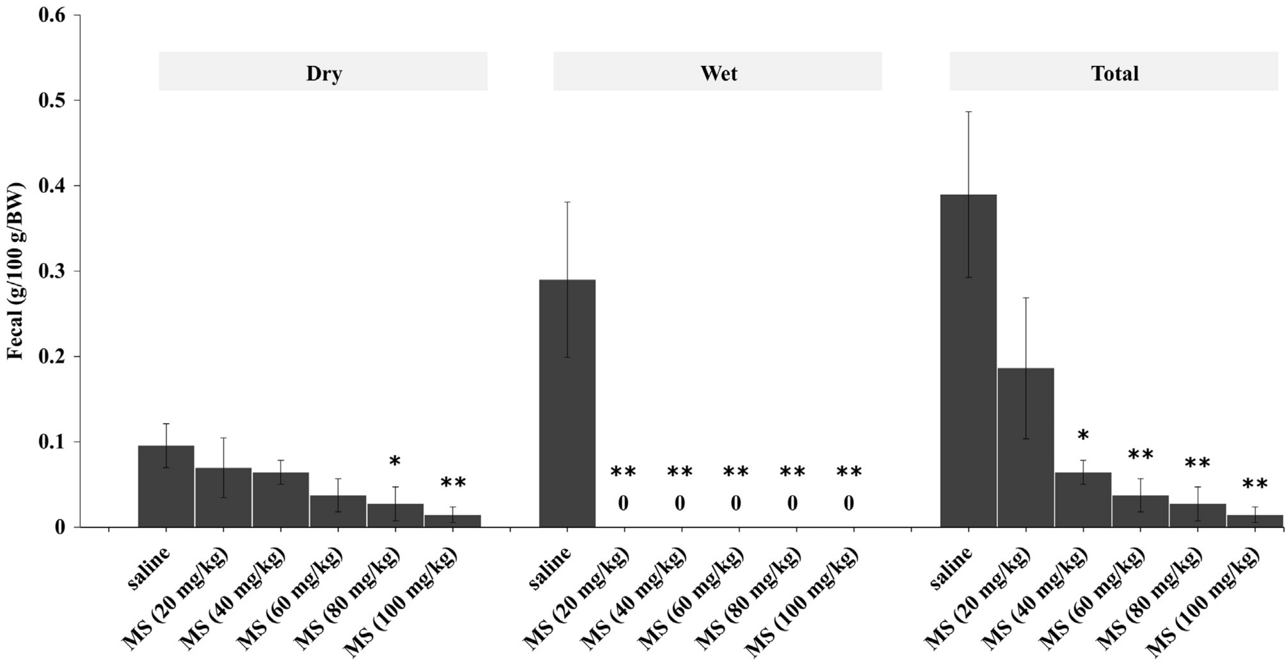

Fig. 2. Effects of *M. speciosa* alkaloid extracts (MS) on dry and wet fecal excretions induced by naloxone-precipitated morphine withdrawal (n = 10–12). Weights of fecal materials excreted during a 30-min period of morphine withdrawal were measured. Data are expressed as means ± S.E.M. *, **, $^{***}P < 0.05$, 0.01, 0.001, respectively, for comparisons with control levels.

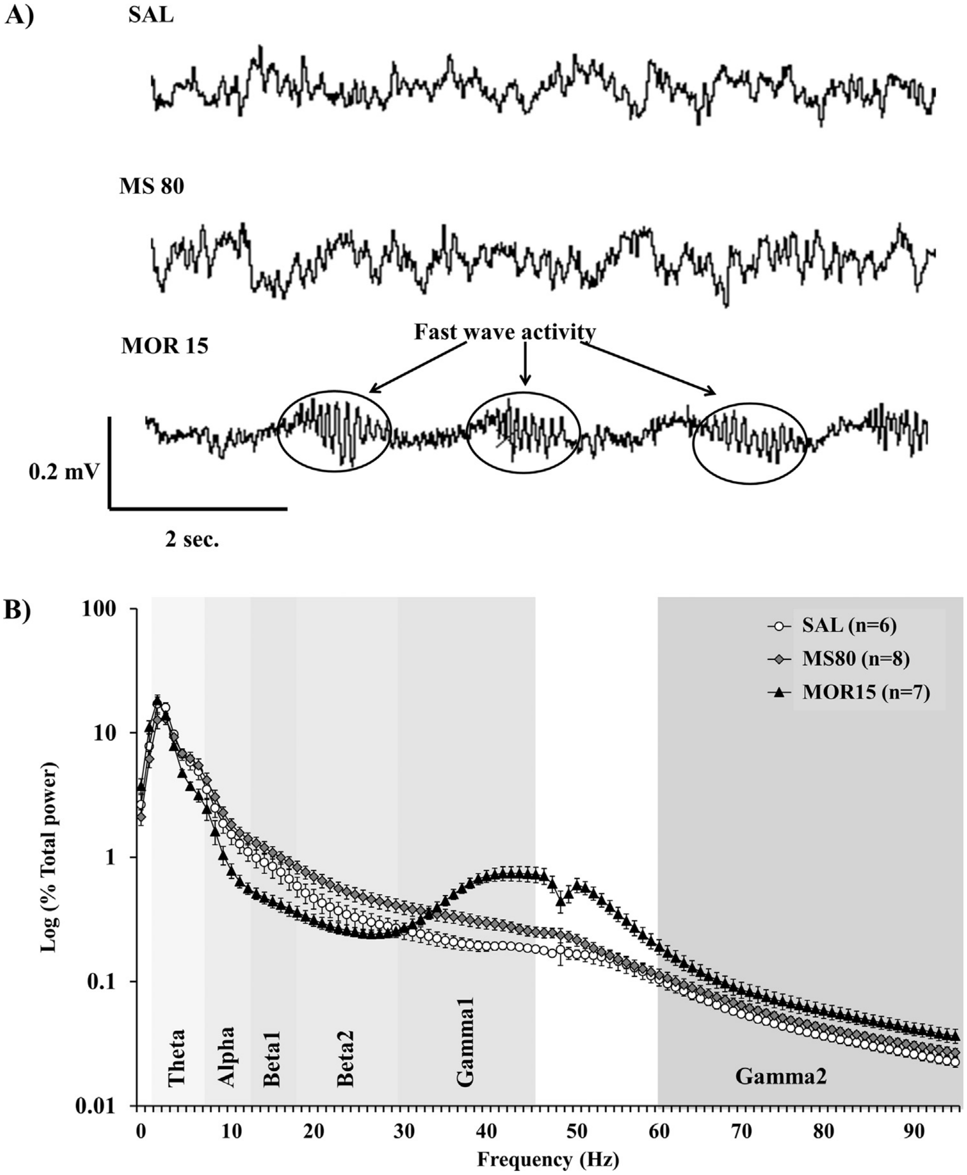

Fig. 3. Patterns of local field potentials following administrations with *M. speciosa* alkaloid extract and morphine (n = 6–8). Time domain of raw LFP signals recorded from the nucleus accumbens of representative mice that received saline (SAL), 80 mg/kg *M. speciosa* alkaloid extract (MS80) and 15 mg/kg morphine (MOR15) are displayed (A). Power spectrums of LFP are expressed in frequency domain (B).

Spectral powers in discrete frequency bands of each group were averaged and expressed either in time or frequency domains.

2.10. Recording and analysis of locomotor activity

Animal behaviors and locomotor activity were recorded using a webcam vertically mounted on the top of recording chamber. Animal image was continuously captured and transferred to the computer. Animal movement was analyzed by using visual C^{++} -based software specifically developed for locomotor activity research (Cheaha et al., 2015a; Reakkamnuan et al., 2015). Basically, translocation of animal was detected with a sensitivity at 2-mm threshold. One count was considered from 1 period of continuous translocation and 1 stop. Levels of locomotor activity were expressed as mean ± S.E.M. of locomotor count per time period.

2.11. Statistical analysis

Experimental data were expressed as mean values ± S.E.M. of the numbers of jumps and weights of fecal materials and urine during a withdrawal period. Differences were determined using one-way analysis of variance (ANOVA), followed by multiple comparisons using Turkey's Method. Differences with $P \leq 0.05$ were considered statistically significant.

3. Results

3.1. Effects of M. speciosa alkaloid extract on jumping behavior induced by naloxone-precipitated morphine withdrawal

Naloxone-induced jumping behavior in morphine-dependent mice was rapidly observed following naloxone injection. Mostly, the numbers of jumping increased from the first minute and peaked in the second minute (Fig. 1A). Jumping numbers gradually decreased to almost zero within the 5th minute and disappeared thereafter. Therefore, jumping behavior during a 5-min period was counted and analyzed to represent the intensity of morphine withdrawal for all groups (Fig. 1B). Jumping number of the control animals (46.2 ± 3.0) was used for comparison with data of other groups treated with either *M. speciosa* alkaloid extract or pure mitragynine. One-way ANOVA revealed suppressive effects seen in treated groups that received *M.*

speciosa alkaloid extract. Multiple comparisons confirmed that jumping number induced by naloxone-precipitated morphine withdrawal was significantly decreased by *M. speciosa* alkaloid extract at 60, 80 and 100 mg/kg in a dose-dependent manner. However, no significant difference was seen in treated groups that received pure mitragynine (30, 90 and 120 mg/kg).

3.2. Effects of M. speciosa alkaloid extract on diarrhea induced naloxone-precipitated morphine withdrawal

Defecation was evaluated from dry and wet fecal materials. Data were expressed as weight per 100 g body weight (Fig. 2). One-way ANOVA showed that treatment with different doses of the *M. speciosa* alkaloid extract significantly reduced dry fecal weight compared to the saline control (p = 0.001). The effect of the *M. speciosa* alkaloid extract was more obvious in the analysis of the wet feces. The saline control showed 0.29 ± 0.09 g per 100 g body weight (BW) of wet fecal materials compared to zero in all treated groups. The analysis of total fecal materials also confirmed significant effect of *M. speciosa* alkaloid extract on defecation during morphine withdrawal period (p = 0.001).

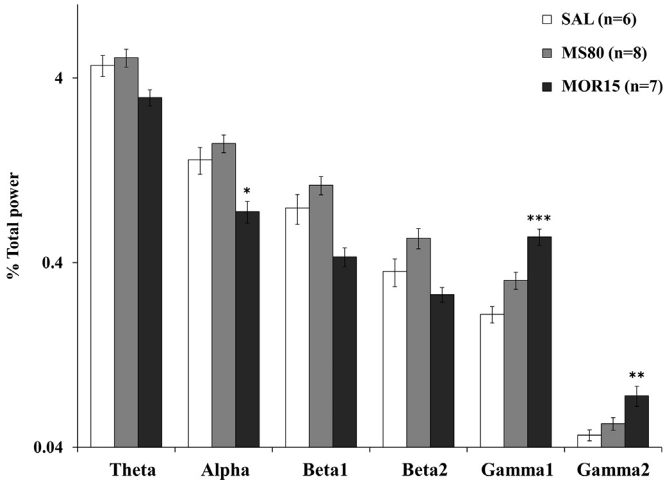

Fig. 4. Effects of administration with saline (SAL), 80 mg/kg *M. speciosa* alkaloid extract (MS80) and 15 mg/kg morphine (MOR15) on powers of 6 discrete frequency ranges (n = 6–8). Averaged percent total powers of different frequency ranges are expressed as means ± S.E.M. *, **, *** $P < 0.05, 0.01, 0.001$, respectively, compared with control levels (one-way ANOVA followed by Tukey's post hoc test).

3.3. Effects of M. speciosa alkaloid extract and morphine treatment on local field potential in the nucleus accumbens

LFP signals from individual mice were continuously recorded for 3 h following treatment with either vehicle, *M. speciosa* extract (80 mg/kg BW, p.o.) or morphine (15 mg/kg BW, i.p.). Representative raw LFP tracings are shown in time domain (Fig. 3A). By visual inspection, relatively similar patterns were seen between LFPs of control and *M. speciosa* alkaloid extract but not morphine group. The latter group appeared to have additional oscillations of fast wave superimposed in slow waves of raw LFP signals. Therefore, all signals were converted into frequency domain for LFP powers analysis (Fig. 3B). Data were illustrated in LFP power spectra (% total power) for 1–100 Hz frequency range. Obviously, morphine appeared to produce different pattern of LFP power spectrum from that of vehicle group. Morphine seemed to reduce slow frequency powers particularly below 30 Hz and increase powers of frequencies above 30 Hz. However, *M. speciosa* alkaloid extract did not change LFP powers throughout the frequency range analyzed. The LFP spectrum produced by *M. speciosa* extract was relatively similar to that of vehicle group. Finally, broad power spectra were divided and assigned into 6 discrete frequency ranges for statistical analysis. One-way ANOVA revealed significant changes in theta, alpha, beta1, beta2, low gamma and high gamma (Fig. 4). Multiple comparisons also indicated significant decreases in alpha and increases in low gamma and high gamma powers produced by morphine treatment. No significant difference between powers of vehicle and *M. speciosa* alkaloid extract groups was found.

Therefore, time-course effect of treatments on low gamma power was particularly analyzed (Fig. 5). Following morphine treatment, low gamma power was rapidly increased and peaked approximately within 40 min. Therefore, the effect of morphine gradually decreased to

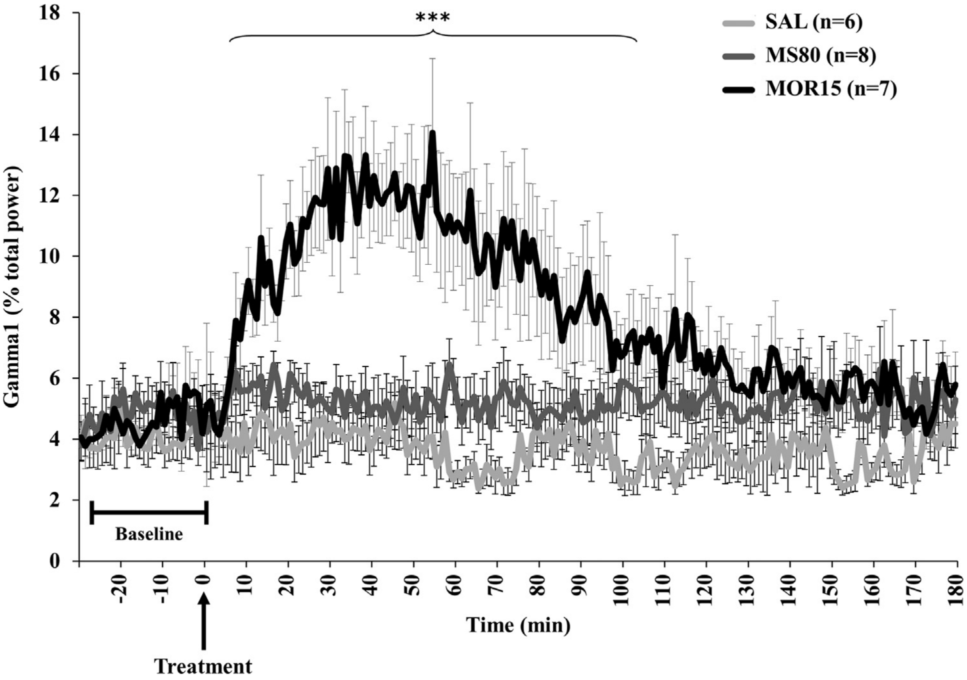

Fig. 5. Average percent total power of gamma1 range were analyzed every 5 min following administration with saline (SAL), 80 mg/kg *M. speciosa* alkaloid extract (MS80) and 15 mg/kg morphine (MOR15) (n = 6–8). Data were compared with control levels using one-way ANOVA followed by Tukey's post hoc test. *, **: $P < 0.05, 0.01$, respectively.

control level within 3 h. Statistical analysis also confirmed significant effect of morphine treatment on low gamma power. On the other hand, no significant change was produced by *M. speciosa* alkaloid extract administration.

3.4. Effects of M. speciosa alkaloid extract on spontaneous motor activity

Levels of spontaneous motor activity were counted every 5 min (Fig. 6). Locomotor count was progressively increased following morphine treatment. The levels of locomotor activity peaked within 60 min and decreased to baseline level within 2.5 h. No significant effect of *M. speciosa* alkaloid extract on spontaneous motor activity was seen.

4. Discussion

The present study demonstrated that the alkaloid extract from *Mitragyna speciosa* (*M. speciosa*) significantly attenuated the intensity of morphine withdrawal symptoms. Particularly, *M. speciosa* alkaloid extract clearly reduced jumping behavior induced by morphine withdrawal. Jumping is a sign frequently used to evaluate morphine dependence in a mice model (Ballard and McAllister, 1999; Reddy and Kulkarni, 1997). It is also one of central withdrawal symptoms that may reflect craving. On the other hand, mitragynine did not reduce number of jumping. These data indicate that some minor ingredients of this plant may have more promising properties especially for this purpose.

Among various constituents of *M. speciosa*, 7-hydroxymitragynine, one of minor alkaloids of this plant, exhibited a more potent antinociceptive effect than even morphine (Matsumoto et al., 2004b). This may explain why *M. speciosa* alkaloid extract produced effective properties whereas mitragynine did not. Moreover, apart from mitragynine and 7-hydroxymitragynine, there might be some other biologically active minor constituents in this plant that act on transmitter systems and result in attenuation of morphine withdrawal symptoms. Thus, the combination of many constituents included in *M. speciosa* alkaloid extract is likely to produce stronger therapeutic effects.

In traditional use, chewing kratom leave and swallowing its crude extract are also known for total alkaloid constituents. In general, biological activities of kratom leaves rely mostly on the alkaloid constituents. The extraction in this study was performed to obtain crude alkaloid extract containing total alkaloid constituents. In terms of confirmation, the crude alkaloid *M. speciosa* extract was analyzed with HPLC and LC-MS to determine the major constituents (Supp. Figs. 1 and 2). These reports suggest that treatment with the crude *M. speciosa* extract can supply relatively more or equal alkaloids to that of chewing process.

Another highlight of the present study was the effect of *M. speciosa* alkaloid extract on diarrhea during morphine withdrawal period. Diarrhea represents peripheral symptom that reflects the level of intestinal motility during a morphine withdrawal period. The suppressive effect of the *M. speciosa* alkaloid extract on morphine withdrawal-induced diarrhea was correlated with previous report of the *in vitro* study using smooth muscle (Watanabe et al., 1997). It has long been known that people use opium to stop diarrhea. Consistently, both synthetic agents and natural product extracts that act as opioid receptor agonists also have an antidiarrheal action (Broccardo and Improta, 1992; Shook et al., 1989). Taken together, these data highlighted that *M. speciosa* alkaloid extract completely suppressed diarrhea and attenuated behavioral hyperactivity that might represent the intensity of craving during a period of withdrawal. In terms of mechanism, serotonergic neurotransmitter system has been particularly involved in attenuation of morphine withdrawal severity. Pre-injection with antidepressant drugs such as fluoxetine, clomipramine, or citalopram, significantly attenuated the naloxone precipitated syndrome (Wu et al., 2005). Additionally, fluvoxamine and sertraline, selective serotonin reuptake inhibitors, also reduced the severity of the naloxone precipitated opioid withdrawal syndrome (Gray, 2002). These findings suggest that the *M. speciosa* alkaloid extract might attenuate morphine withdrawal-induced jumping behavior partly through its antidepressant-like activity.

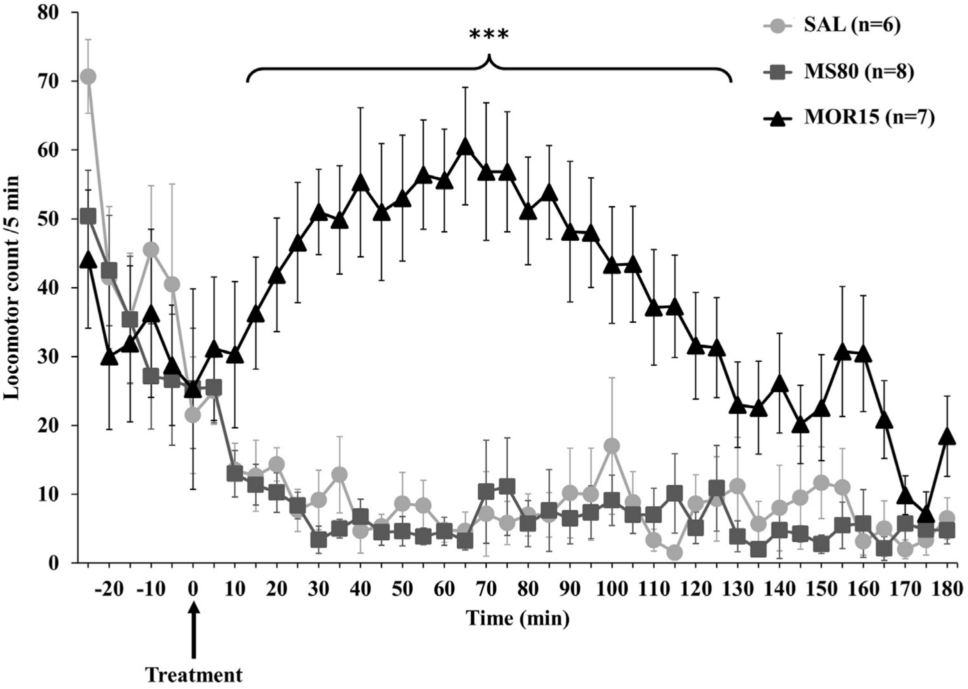

Fig. 6. Effects of administration with saline (SAL), 80 mg/kg *M. speciosa* alkaloid extract (MS80) and 15 mg/kg morphine (MOR15) on spontaneous motor activity (n = 6–8). Locomotor counts in each group were averaged 5 min during baseline period and following the administration for 3 h. Data are expressed as means ± S.E.M. *** $P < 0.001$ for comparisons with control levels (one-way ANOVA followed by Tukey's post hoc test).

Activation of dopaminergic brain pathways and spontaneous motor activity by drugs of addiction are well-known. Particularly, activation of the nucleus accumbens and striatum by psychostimulants and opiates is believed to play a major role in the establishment of drug dependence and withdrawal phenomena (Hyman, 1996; Koob, 1996; Nestler, 1996). Similar findings were seen following injections of cocaine and methamphetamine (Wang and McGinty, 1995; Young et al., 1991). In term of behavioral output, psychostimulants also enhance locomotor activity (Vezina, 2004). These are among the most common effects produced by addictive substances. However, *M. speciosa* alkaloid extract did not activate the nucleus accumbens and spontaneous motor activity at all. The present data may explain why kratom use for emotional purpose has not been reported. Therefore, the impact of situations and context like social, ritualistic or self-medicating factors may be discussed why kratom consumption is still widespread in some countries.

Until now, methadone (Wang et al., 2015) and levo-α-acetylmethadol (LAAM) (Judson and Goldstein, 1979) are routinely used in pharmacological intervention for opiate dependence. Continuous use of these agents gives benefits in suppressing opioid withdrawal symptoms for long durations. However, some patients also reported experiencing withdrawal symptoms during treatment (Dyer and White, 1997). Increasing the dose of methadone has benefits in suppressing withdrawal symptoms but also leads to some risks both physically and psychologically (Kreek, 1973). Rapid transition to dependence were associated with anxiety disorders in methadone maintained patients (Karsinti et al., 2016). In particular, methadone maintained patients were found to have difficulty in learning the outcomes of positive stimulus in drug-related context (Levy-Gigi et al., 2014). This finding was believed to explain why many opiate users relapse back to using opiates over and over again throughout their lives.

Mechanisms of opioid withdrawal were found to involve various neurotransmitter systems including opioid and non-opioid systems (Freye and Latasch, 2003; Gulati et al., 2004; Puppala et al., 2006; Williams et al., 2001). These might explain why opioid alone is insufficient for morphine withdrawal treatment. Additional treatments with agonists of other neurotransmitter systems are highly recommended. The crude alkaloid extract is likely to be suitable for opioid withdrawal treatment having more constituents to produce broader spectrum of CNS actions. Therefore, alternative treatments are needed to substitute methadone or to be used in combination with a small dose of methadone to enhance treatment efficacy. According to our findings, the *M. speciosa* alkaloid extract might be used as an alternative treatment in combination with other effective opioid agonists. CNS action of *M. speciosa* alkaloid extract is unlikely to mimic that of opiates according to LFP patterns and levels of spontaneous motor activity.

Conflict of interest

All authors declared that they have no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jep.2017.07.008.

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